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Effects of High Temperature and Disinfectants on the Viability of *Sarcocystis neurona* Sporocysts

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ABSTRACT: The effect of moist heat and several disinfectants on *Sarcocystis neurona* sporocysts was investigated. Sporocysts (4 million) were suspended in water and heated to 50, 55, 60, 65, and 70 C for various times and were then bioassayed in interferon gamma gene knockout (KO) mice. Sporocysts heated to 50 C for 60 min and 55 C for 5 min were infective to KO mice, whereas sporocysts heated to 55 C for 15 min and 60 C or more for 1 min were rendered noninfective to mice. Treatment with bleach (10, 20, and 100%), 2% chlorhexidine, 1% betadine, 5% *o*-benzyl-*p*-chlorophenol, 12.56% phenol, 6% benzyl ammonium chloride, and 10% formalin was not effective in killing sporocysts. Treatment with undiluted ammonium hydroxide (29.5% ammonia) for 1 hr killed sporocysts, but treatment

TABLE I. Survival of *Sarcocystis neurona* sporocysts at 50–70 C exposed for 1–60 min.

Temperature (C)	Exposure time (min)	No. of mice positive for *S. neurona*/no. of mice fed	Time to illness, death
50	2	2/2	18, 19
	10	2/2	19, 19
	30	2/2	20, 20
	60	2/2	20, 20
55	1	2/2	20, 20
	2	2/2	24, 24
	5	2/2	35, 35
	15	0/2	
	30	0/2	
60	1	0/4*	
	2	0/4*	
	5	0/2	
65	1	0/4*	
	2	0/4*	
70	1	0/2	
	2	0/2	

* Repeat experiments.

with a 10-fold dilution (2.95% ammonia) for 6 hr did not kill sporocysts. These data indicate that heat treatment is the most effective means of killing *S. neurona* sporocysts in the horse feed or in the environment.

Sarcocystis neurona is the most important cause of a neurologic disease of horses in the Americas (Dubey, Lindsay et al., 2001). Horses become infected with *S. neurona* by ingesting sporocysts excreted in feces of infected opossums (Fenger et al., 1997; Cutler et al., 2001; Saville et al., 2001). Approximately 50% of the horses in the United States were found to have antibodies to *S. neurona* (Dubey, Lindsay et al., 2001). The high prevalence of antibodies to *S. neurona* indicates widespread exposure of the forage or horse feed to *S. neurona*. Practical methods to kill *S. neurona* in the horse environment are not known. Several studies have reported likely cross-contamination of control horses in experimental settings (Cutler et al., 2001; Saville et al., 2001). The objective of the present study was to test the effects of different high temperatures and of commonly used disinfectants for killing sporocysts.

Sarcocystis neurona sporocysts were obtained from the intestinal homogenate of a laboratory-raised opossum (no. 30) that was fed muscles of an experimentally infected raccoon (Dubey, Saville et al., 2001). The raccoon had been fed *S. neurona* sporocysts 78 days before euthanasia. The opossum intestines were homogenized in water, filtered through a 90-μm metallic sieve, suspended in antibiotic saline, and stored at 4 C, as described previously (Dubey, 2000). The sporocysts had been stored at 4 C for 2–3 wk before use in the present study.

There were approximately 8 million sporocysts per milliliter of the sporocyst pool used in this study. The viability of sporocysts was determined by bioassays in interferon gamma gene knockout (KO) mice (Dubey and Lindsay, 1998). For bioassay, 0.5 ml of the sporocyst pool was diluted 10-fold in saline, and 0.5 ml from each aliquot from the 10^{-3} to 10^{-6} dilutions was fed to each of the 5 KO mice.

To study the effect of exposure to 50–70 C, heat-sealable plastic pouches were used to contain sporocysts so that they were exposed instantly to the desired temperature (Table I). One half milliliter of sporocyst suspension was dispensed into 10 × 15 cm boilable plastic bags (Kapak/Scotchpak, Kapak Corporation, Minneapolis, Minnesota). Each bag was sealed to prevent sporocyst spillage. After stated exposure time, pouches were removed immediately from the water bath to room temperature (22 C) and then bioassayed in 2 KO mice for each treatment. There was immediate cooling after removal from the water bath because the pouches were very thin and the small volume was spread over a large area.

TABLE II. Effects of disinfectants on viability of *Sarcocystis neurona* sporocysts.

Disinfectant and concentration (%)	Total volume	Infectivity to KO mice*
Bleach (Clorox), 5.25% sodium hypochlorite		
100	20	4/4
20	20	4/4
10	20	4/4
Wex-cide, 12.56% phenol		
0.003	20	4/4
Novalsan, 2% chlorhexidine		
24	20	4/4
Betadine, 1% iodine		
100	20	4/4
TB plus, 5% *o*-benzyl-*p*-chlorophenol		
0.003	15	4/4
NPD, 6% benzyl ammonium chloride		
0.007%	20	4/4
Formalin, 10%	20	4/4
Ammonium hydroxide		
100%	20	0/4
10%	20	2/4
Untreated	20	4/4

* Number of mice positive for *S. neurona* of number of mice fed. Data for 1- and 6-hr treatments were pooled.

To study the effect of different disinfectants, 0.5 ml of the sporocyst suspension was mixed with 15 or 20 ml of the disinfectant in 50-ml tubes (Table II). The disinfectants used in this study included Clorox® (The Clorox Company, Oakland, California), Novalsan® (Fort Dodge Animal Health, Fort Dodge, Iowa), Betadine® (The Purdue Frederick Co., Stamford, Connecticut), TB plus® (Betco, Toledo, Ohio), Wexcide® (Wexford Labs, Inc., Kirkwood, Missouri), NPD® (Covatec/Calgon Vestal Lab., Inc., St. Louis, Missouri), ammonium hydroxide (Sigma, St. louis, Missouri), and 10% formalin. After 1 or 6 hr of incubation at room temperature, the treated sporocysts were mixed well with 25 ml of water and centrifuged at 2,000 rpm (1,200 *g*) for 10 min. After discarding the supernatant, the sediment was mixed with water and centrifuged, and the process was repeated at least 3 times to remove the disinfectants. After a final wash, the materials were fed to 2 KO mice.

The KO mice were examined for *S. neurona* infection. Mice that became ill were killed and their cerebellums examined microscopically for *S. neurona* schizonts and merozoites; the cerebellum is the most heavily parasitized with *S. neurona* in mice examined 25 days after feeding sporocysts (DAFS) (Dubey, 2001). The survivors were bled and killed 2 mo after feeding sporocysts; their sera were tested for antibodies to *S. neurona* in the agglutination test (Lindsay and Dubey, 2001), and their brains were examined immunohistochemically (Dubey and Hamir, 2000). Mice were considered infected when *S. neurona* was demonstrated in their tissues. Mice were considered uninfected when antibodies to *S. neurona* and *S. neurona* parasites were not demonstrable.

The mice fed with sporocysts heated to 50 or 55 C for indicated times developed neurologic signs, and *S. neurona* was demonstrable in their brains (Table I). All mice fed sporocysts heated to 55 C for 15 min and 60 C for 1 min or more remained healthy with no demonstrable *S. neurona* stage or antibody. All control mice fed with untreated sporocysts developed neurologic signs and were killed 21–28 DAFS; *S. neurona* was found in the brains of all infected mice. These data indi-

TABLE III. Infectivity of untreated *Sarcocystis neurona* sporocysts to KO mice.

Dilution of inoculum	No. of mice fed	Day of death or euthanasia
10^{-3}	5	30, 30, 30, 31, 31
10^{-4}	5	23, 25, 28, 28, 31
10^{-5}	5	23, 31, 36, S, S*
10^{-6}	5	39, S, S, S, S

* S = Survived, no antibody, no parasites.

cate that heating horse feed to 60 C should kill *S. neurona* sporocysts and steaming the contaminated environment (e.g., horse barn) should be effective in killing *S. neurona* sporocysts.

None of the disinfectants tried was effective in killing *S. neurona* sporocysts, except ammonia (Table II); the mice died of neurologic *S. neurona* infection. The concentrations selected were those advised on the product-label. Exposure to concentrated ammonia killed sporocysts, but diluted ammonia was not effective in killing all sporocysts (Table II).

All 10 mice fed with 10^{-3} and 10^{-4} dilutions, 3 out of 5 fed with the 10^{-5} dilution, and 1 of 5 mice fed with the 10^{-6} dilution died of *S. neurona* infection, indicating that there were at least 100,000 viable sporocysts used for each treatment (Table III).

Little is known of the pathogenesis of *S. neurona* infection in horses. All attempts to fulfill Koch's postulates in horses have been unsuccessful. Horses fed with *S. neurona* sporocysts developed mild or moderate neurologic signs, sometimes associated with microscopic lesions but without demonstrable organisms. There are many other difficulties in attempting to induce *S. neurona* infection in horses. First, it is difficult to locate *S. neurona*–negative horses. Second, some of the sporocysts fed to horses pass unexcysted in feces; thus, they are potentially infective to control horses (Cutler et al., 2001; Saville et al., 2001). Results of the present study indicate that to prevent tracking of sporocysts between barns or stalls, it is necessary to change boots or use disposable boot covers because the common practice of using disinfectant footbaths to prevent cross-contamination will not kill sporocysts. In addition, steam cleaning of equine facilities will likely be useful to kill the sporocysts in between animal use.

LITERATURE CITED

CUTLER, T. J., R. J. MACKAY, P. E. GINN, K. GILLIS, S. M. TANHAUSER, E. V. LERAY, J. B. DAME, AND E. C. GREINER. 2001. Immunoconversion against *Sarcocystis neurona* in normal and dexamethasone-treated horses challenged with *S. neurona* sporocysts. Veterinary Parasitology **95:** 197–210.

DUBEY, J. P. 2000. Prevalence of *Sarcocystis* species sporocysts in wild caught opossums (*Didelphis virginiana*). Journal of Parasitology **86:** 705–710.

———. 2001. Migration and development of *Sarcocystis neurona* in tissues of interferon gamma knockout mice fed sporocysts from a naturally infected opossums. Veterinary Parasitology **95:** 341–351.

———, AND A. N. HAMIR. 2000. Immunohistochemical confirmation of *Sarcocystis neurona* infections in raccoons, mink, cat, skunk and pony. Journal of Parasitology **86:** 1150–1152.

———, AND D. S. LINDSAY. 1998. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. International Journal for Parasitology **28:** 1823–1828.

———, D. S. LINDSAY, W. J. A. SAVILLE, S. M. REED, D. E. GRANSTROM, AND C. A. SPEER. 2001. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Veterinary Parasitology **95:** 89–131.

———, W. J. A. SAVILLE, J. F. STANEK, D. S. LINDSAY, B. M. ROSENTHAL, M. J. OGLESBEE, A. C. ROSYPAL, C. J. NJOKU, R. W. STICH, O. C. H. KWOK, S. K. SHEN, A. N. HAMIR, AND S. M. REED. 2001. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): Evidence for natural infection with sarcocysts, transmission of infection to opossums (*Didelphis virginiana*), and experimental induction of neurologic disease in raccoons. Veterinary Parasitology **100:** 117–119.

FENGER, C. K., D. E. GRANSTROM, A. A. GAJADHAR, N. M. WILLIAMS, S. A. MCCRILLIS, S. STAMPER, J. L. LANGEMEIER, AND J. P. DUBEY. 1997. Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis* sp. sporocysts from the opossum (*Didelphis virginiana*). Veterinary Parasitology **68:** 199–213.

LINDSAY, D. S., AND J. P. DUBEY. 2001. Direct agglutination test for the detection of antibodies to *Sarcocystis neurona* in experimentally infected animals. Veterinary Parasitology **95:** 179–186.

SAVILLE, W. J. A., R. W. STICH, S. M. REED, C. J. NJOKU, M. J. OGLESBEE, A. WUNSCHMANN, D. L. GROVER, A. L. LAREW-NAUGLE, J. F. STANEK, D. E. GRANSTROM, AND J. P. DUBEY. 2001. Utilization of stress in the development of an equine model for equine protozoal myeloencephalitis. Veterinary Parasitology **95:** 211–222.